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Packaging of human endogenous retrovirus sequences is undetectable in porcine endogenous retrovirus particles produced from human cells

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Abstract

The chronic shortage of human donor organs and tissues for allotransplantation could be relieved if clinical xenotransplantation were to become a viable clinical therapy. Balanced against the benefits of xenotransplantation are the possible consequences of zoonotic infections, and in particular, infection by porcine endogenous retrovirus (PERV). An often-proclaimed risk of PERV infection is the possible recombination of PERV with human endogenous retroviruses (HERV) (Lee et al., 2002). To address this issue, we examined the potential for HERV sequences to be cross-packaged into PERV particles produced from infected human 293 cells. Although HERV-K, W, E, R, and ERV-9 RNA transcripts are expressed in 293 cells, we did not detect cross-packaging of any of these HERV groups. Quantitative analysis indicated that less than approximately 1 in 10^4–10^7 PERV particles might contain HERV sequences. In comparison, we found that murine leukemia virus (MLV)-based vector transcripts were cross-packaged at a rate of approximately one copy in 10^4 PERV particles. Our results indicate that the potential for recombination of PERV and HERV sequences is low and that novel viruses generated by this mechanism are unlikely to represent a significant risk for xenotransplantation.

Keywords: Xenotransplantation; PERV; HERV; Retrovirus; Recombination; Cross-packaging; Safety

Introduction

Xenotransplantation of porcine organs has the potential to overcome the severe shortage of human tissues and organs available for human transplantation (Sachs et al., 2001). However, this procedure also carries with it a number of important microbiological safety issues relating to the possible infection of a xenotransplant recipient with a zoonotic microorganism (Weiss et al., 2000). Encouragingly, many exogenous microorganisms of pigs can be eliminated from donor animals by the use of barrier derivation procedures (Tucker et al., 2002). In contrast, because porcine endogenous retrovirus (PERV) is part of the germ line DNA of pigs and is therefore unaffected by such procedures, its control is more challenging. In this article we investigate a possible consequence resulting from the infection of a human cell by PERV, namely, the potential generation of novel infectious viruses via the cross-packaging and recombination of PERV and human endogenous retrovirus (HERV) sequences.

Endogenous retroviruses (ERVs) are present in the genomes of all vertebrates. They are a diverse group of elements whose gene organization is very similar to that of exogenous retroviruses (Boeke and Stoye, 1997; Stoye, 2001; Wilkinson et al., 1994). At the nucleotide level, ERVs can have homology to γ-retroviruses (e.g., murine leukemia viruses, MLV) (Akiyoshi et al., 1998; Armstrong et al., 1971; Patience et al., 2001; Wilkinson et al., 1994), as well as β-retroviruses (e.g., mouse mammary tumor virus) (Ericsson et al., 2001; Patience et al., 2001; Wilkinson et al., 1994), but share little or no homology to lentiviruses (e.g., human immunodeficiency virus) (Horwitz, et al., 1992). Although multiple copies of ERV are present in the genome of their host, the majority of these loci are not replication competent due to point mutations or deletions disrupting one or more of the three main open reading frames (ORFs); *gag*, *pol*, and *env* (Wilkinson et al., 1994). Nevertheless, some ERV loci have retained the ability to encode one or

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more viral proteins and can, on occasion, produce infectious particles. PERV is one example of an infectious ERV (Patience et al., 1997; Wilson et al., 1998).

The genomic DNA of pigs contains at least 13 groups of PERV that are related to γ- and β-retroviruses (Ericsson et al., 2001; Klymiuk et al., 2002; Patience et al., 2001). Infectious PERV are thought to be limited to only one of these groups, as members of the remaining groups have extensive ORF disruptions (Ericsson et al., 2001; Klymiuk et al., 2002; Patience et al., 2001). Within this group, three infectious PERV subgroups have been identified, PERV-A, -B, and -C (Takeuchi et al., 1998). PERV-A and -B can infect human and porcine cells (Takeuchi et al., 1998). PERV-C is more limited in its tropism and can infect only porcine cells (Oldmixon et al., 2002; Takeuchi et al., 1998). Recombination between PERV can occur and can result in the formation of high-titer PERV isolates (Oldmixon et al., 2002; Wilson et al., 2000). Although PERV is infectious for certain human cells in vitro, no infection of xenotransplant recipients following clinical transplantation has been detected (Dinsmore et al., 2000; Elliott et al., 2000; Heneine et al., 1998; Paradis et al., 1999; Patience et al., 1998a).

The human genome also contains ERVs related to γ- and β-retroviruses (Wilkinson et al., 1994). All HERV loci identified to date possess at least one defective ORF and are therefore not infectious (Tristem, 2000). However, some HERV groups such as HERV-E, HERV-R, ERV-9, HERV-H, HERV-W, and HERV-K possess either complete or near complete ORFs for one or more of their component genes and are also widely expressed in primary human cells (Wilkinson et al., 1994). If human cells were to become infected by PERV, these HERV groups would be the most likely substrates for the generation of novel recombinant viruses.

To date, the risk of HERV recombination with PERV has been the subject of debate, but little research. Theoretically, HERV–PERV recombination could occur during the integration of PERV into the human genomic DNA following the infection of a cell by PERV. Much more likely is the generation of HERV–PERV recombinants during a productive infection following the copackaging of a HERV and PERV genomic transcript into an individual retrovirus particle (Telesnitsky and Goff, 1997). Thus, the demonstration of cross-packaging of HERV sequences into PERV particles would reflect an increased probability of recombination between these viruses, and ultimately, an increased potential for the generation of chimeric viruses with unpredictable pathogenicities. In this article we examine the potential for HERV transcripts to be packaged into PERV particles using a sensitive in vitro assay system.

Results

Comparison of PERV replication in human cell lines

To maximize the potential to detect cross-packaging of HERV sequences by PERV, the human cell line must support PERV replication to high titer. To date, only three human cell lines have been identified that support PERV replication: 293 cells (Patience et al., 1997), HepG2 cells (Wilson et al., 2000), and to a lesser extent HeLa cells (Wilson et al., 2000). We compared PERV replication in 293 and HepG2 cells using a PERV-A isolate that grows to high titers in vitro (PERV-A 14/220) (Oldmixon et al., 2002; Sachs et al., 1976). PERV-A 14/220 is a PERV-A/PERV-C recombinant isolate, identified during an in vitro transmission assay using PBMC from Massachusetts General Hospital major histocompatibility complex (MHC)-inbred miniature swine (Oldmixon et al., 2002; Sachs et al., 1976). We found that the culture supernatant of 293 cells infected with PERV-A 14/220 contained approximately 200-fold more RT activity than the equivalent HepG2 supernatant (55,000 and 230 mU/ml, respectively). These results indicate that based on the greater production of PERV particles, 293 cells are more appropriate than HepG2 cells for the study of HERV cross-packaging by PERV.

HERV expression in human cells

To most stringently examine the potential for HERV–PERV recombination, it is essential that (1) the RT-PCR method is capable of detecting transcripts from as many individual loci within a HERV family as possible and (2) the PERV-infected human cell line expresses a wide spectrum of HERV sequences. We investigated the HERV expression profile in 293 and HepG2 cells by RT-PCR and Northern blot. The sensitivity of the RT-PCRs was maximized using primers designed toward conserved sequences within a number of biologically significant HERV groups (Table 1). A phylogenetic comparison of these HERV groups is presented in Fig. 1A. We found that 293 cells express HERV-E, HERV-R, ERV-9, HERV-H, HERV-W, and HERV-K RNA transcripts at levels comparable to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Fig. 1B). By Northern blot, we found that 293 cells express multiple transcripts for each HERV family (Fig. 1C). Although HepG2 cells expressed RTVL-H and ERV-9 sequences at similar levels to 293 cells, expression of the remaining HERV groups in HepG2 cells was either lower than 293 cells (HERV-E and HERV-W) or was not detected (HERV-R) (data not shown). Thus, based on their broader HERV expression profile, 293 cells appeared more suitable than HepG2 for the study of HERV cross-packaging by PERV.

Analysis of PERV particles for presence of HERV RNA

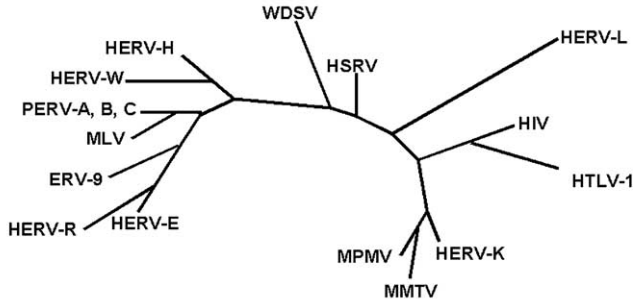
We concentrated and purified PERV particles from the supernatant of 293/PERV-A cells using sucrose density gradient centrifugation. The RT activity profile of the gradient is shown in Fig. 2A and demonstrates that the RT activity of the peak gradient fraction was increased approximately 200-fold, i.e., 1.9×10^6 mU/ml for the peak gradient fraction in comparison to 5.5×10^3 mU/ml for un-

Table 1
Primers and PCR conditions used to detect HERV and PERV sequences

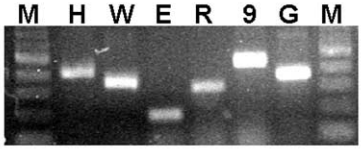
HERV group (gene)	GenBank Accession No.	Sense primer, antisense primer
PERV (Pol)	AF038599	TGCAGGAAACCTCGAGACTC, TAACGTGGGATGCATGGATC
HERV-H (Pol)	K01891	CCTCACCCTGATCACRYTTG, GAATAATGTCTGACAGAAGGG
HERV-W (Pol)	AF009668	AATTATCCAAAGGCACCAGGG, TGTGTAAGGACTCCTAGAGC
HERV-E (Pol)	M10976	TGGCAACTAAGTGGCTACAGG, ATTTGTAGTCTTTTGAGCCCC
ERV-9 (Pol)	X57147	CCTGAAGCTCATAAAGGATTAC, CATCTGAAGGAACAGAGTGCC
HERV-R (Env)	M12140	TGCCAGATAGTATCCATGGG, TCTTCATCGCTGACTCGTGC
HERV-K (Pol)	M14123	CCAGGTTTCAGTGGAAAGTG, GTCTCCTCTTAAGATAGAGAAC
*Lac*Z		CTCTGGCTCACAGTACGCGTAGG
		CCATCAATCCGGTAGGTTTTCCG

concentrated culture supernatant. Most PERV particles banded at a density of approximately 1.14–1.17 g/ml, i.e. a value consistent with mature PERV particles and other C-type retroviruses (Tacke et al., 2000).

A)



B)



C)

HERV group	Transcript length (Kbp)
HERV-H	11, 6, 3.2, 2, 0.3
HERV-W	8, 3
HERV-E	9, 7, 5, 4, 3
HERV-R	9, 5, 3.2, 2.3, 0.5
ERV-9	2
HERV-K	9, 5, 3.2. 1.8

Fig. 1. HERV profile of 293 cells. (A) Phylogenetic comparison of HERV and PERV sequences. (B) HERV expression in 293 cells. Lanes: M, marker; H, HERV-H; W, HERV-W; E, HERV-E, R, HERV-R; 9, ERV-9; G, GAPDH; M. RT-PCR for each HERV group was performed using a random-primed cDNA prepared from 293 cell RNA (Trizol, Invitrogen Life Technologies). The absence of contaminating DNA in the RNA preparation was confirmed by PCR analysis of RNA that had not been subjected to reverse transcription. HERV-K expression was also detected in a separate experiment (data not shown). (C) Multiple HERV RNA transcripts are expressed in 293 cells. The RNA transcript profile of 293 cells was determined by Northern blot using high-stringency wash conditions to maintain probe specificity.

To test for HERV transcripts in these PERV particles, we synthesized a random-primed cDNA from the RNA of pooled peak fractions (spanning the density range 1.14–1.17 g/ml) and analyzed this cDNA by RT-PCR. While high levels of PERV RNA were present in the peak fractions, in marked contrast, HERV RNAs were not detected (Fig. 2B). PERV is known to be capable of cross-packaging MLV-based vectors (Takeuchi et al., 1998). Therefore, for comparison, we examined the ability of PERV particles to cross-package the MLV-derived β-galactosidase (*Lac*Z) reporter vector MFGnlsLacZ. We transduced 293/PERV-A cells at high m.o.i. with MFGnlsLacZ vector and purified particles from the supernatant of these cells by sucrose gradient centrifugation. In contrast to our results with HERV, we detected significant levels of MLV-based vector transcripts in the PERV particles (Fig. 2C).

Determination of the ratio of PERV:HERV sequences in PERV particles

To determine the ratios of PERV:HERV and PERV:MLV-vector transcripts present in PERV particles, we determined the concentration of HERV, PERV, and vector RNAs present in the peak gradient fraction. Having determined the dilution to which the peak fraction RNA could be taken before becoming undetectable by RT-PCR (Fig. 2C), we then determined the sensitivity of the individual RT-PCR reactions using in vitro transcribed RNAs (Table 2). It is noteworthy that while typical sensitivities of the RT-PCRs were approximately 1 pg per reaction, the assays for PERV and HERV-K were less sensitive (detecting approximately 0.1–1.0 ng per reaction). From these results, we were able to calculate the concentration of RNAs present in the peak fraction. For example, RNA from the peak gradient fraction could be diluted approximately 1024-fold before PERV became undetectable by RT-PCR (Fig. 2C). As the sensitivity of the PERV *pol* RT-PCR was 1.25 ng per reaction, we calculated that the peak fraction contained 1.28 μg of PERV RNA (Table 2). Similarly, we calculated that the peak fraction contained 100 pg of MLV vector RNA, and therefore, a *Lac*Z:PERV ratio of approximately 1:38,000. We calculated that $<$1:51,000 (for HERV-K) down to $<$1:1,000,000 (for the remaining HERV families)

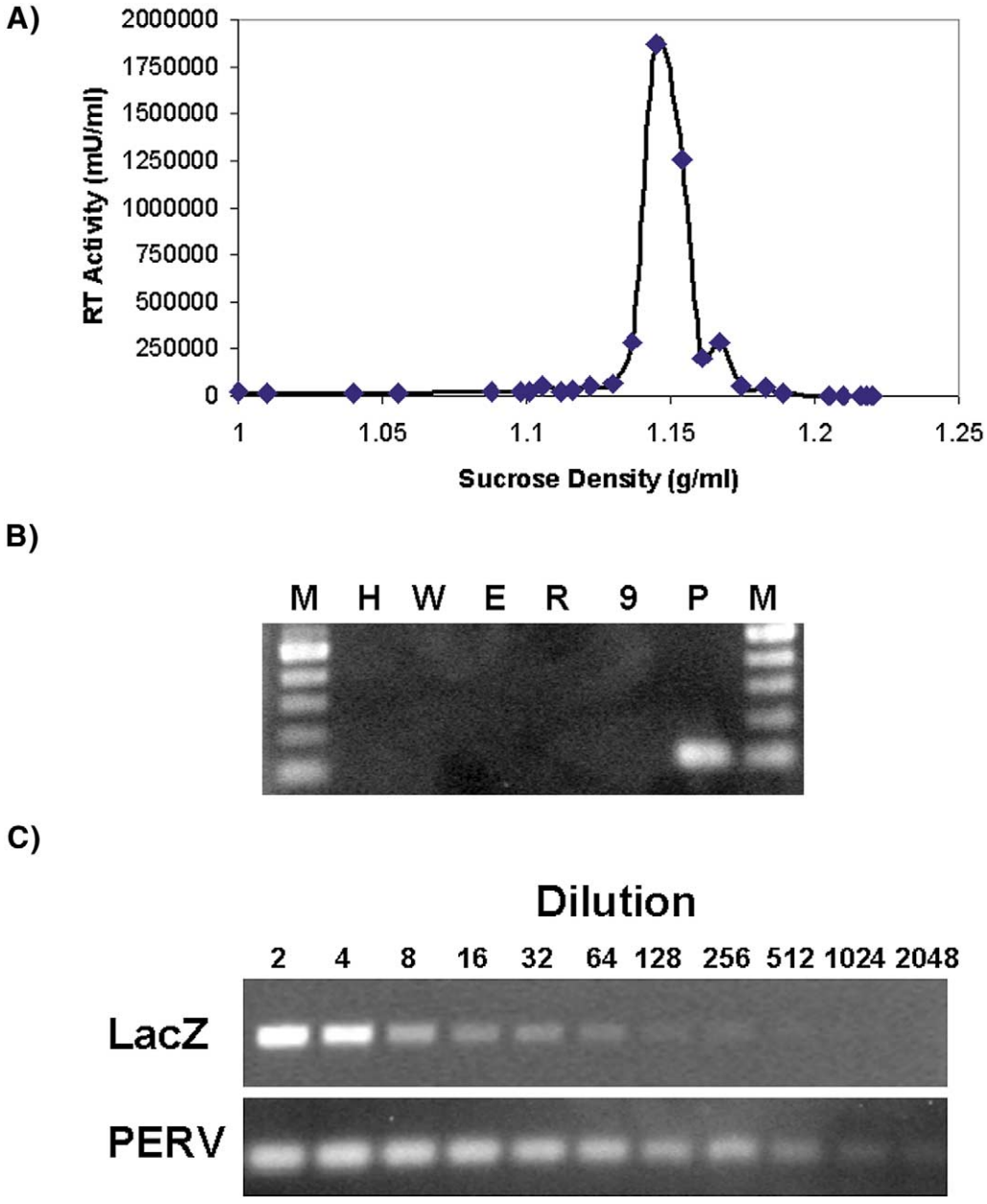


Fig. 2. Analysis of sucrose gradient-purified PERV particles. (A) RT activity of sucrose gradient-purified 293/PERV-A supernatant. (B) RT-PCR for PERV and HERV sequences in peak gradient fractions produced from 293/PERV-A cells. (C) RT-PCR for PERV *pol* and MLV-based vector transcripts using dilutions of RNA purified from the peak sucrose-gradient fraction of 293/PERV-A cells and 293/PERV-A/LacZ cells. PERV production from these cell lines was comparable, i.e., within three-fold according to PERV *pol* RT-PCR (data not shown). No amplicon for PERV *pol* was detected at a 1:4096 dilution of the gradient RNA.

PERV particles contained a HERV transcript (Table 2). Minimum concentrations and ratios of the transcripts are presented because HERV RNAs were not detected.

Discussion

In this article we examine the potential for recombination between PERV and HERV following cross-packaging. Demonstrable cross-packaging might reflect an increased potential for the generation of chimeric viruses. The clinical consequences of such recombination are unpredictable, but could, in a worst case scenario, generate novel viruses with potential to cause human disease.

We designed an in vitro system to investigate HERV–PERV recombination based on the assumption that the potential for recombination is greatest if two distinct retroviral transcripts are copackaged into individual virus particles. Therefore, to provide as diverse an analysis as possible, we required that the infected human cell line used in this study should (1) express a broad spectrum of HERV and (2) be productively infectable by PERV. We found that 293 cells

Table 2
Quantification of ERV sequences present in PERV particles produced from 293 cells

Retroviral transcript	RT-PCR sensitivity (pg/μl)[a]	Peak fraction RNA concentration (pg/μl)	HERV:PERV ratio[b]
PERV	1250	1,280,000	NA
HERV-H	0.625	<0.625	<1:8,200,000
HERV-W	1.25	<1.25	<1:3,900,000
HERV-E	0.312	<0.312	<1:8,200,000
HERV-R	0.625	<0.625	<1:6,400,000
ERV-9	5.0	<5.0	<1:6,100,000
HERV-K	100	<100	<1:51,000
*Lac*Z	0.039	100	1:38,400

NA, not applicable.

[a] Minimum input of transcribed standard RNA that resulted in a positive RT-PCR signal.

[b] Note that only minimum ratios can be presented because HERV RNAs were undetectable in the peak fractions. All ratios take into account the sizes of the individual RT-PCR amplicons.

expressed a wide range of HERV transcripts, including long transcripts of HERV-H, -W, -E, -R, -K, and ERV-9. The expression of long transcripts is noteworthy as these are most likely to contain intact packaging sequences and therefore be candidates for cross-packaging into PERV particles. Our detection of HERV-R expression in 293 cells was somewhat unexpected as this HERV was initially thought to have an expression profile restricted to cells of placental and embryonic origins (Boyd et al., 1993). However, more recently, expression has also been detected in additional tissue types (Andersson et al., 1998; Katsumata et al., 1998; Sibata et al., 1997).

In addition to its broad HERV expression profile, we chose the 293 cell line as our prototypic human cell because it is the most permissive cell line for replication of PERV that has been identified to date. In this regard, although many other human cell lines and primary cells express receptors for PERV and are also susceptible to PERV pseudotypes, replication of PERV in these cells is either extremely low or nonproductive (Patience et al., 1997; Takeuchi et al., 1998; Wilson et al., 2000). Currently, the block to PERV replication in these cells is not known. It would prove interesting to expand the studies of HERV–PERV copackaging into additional cell lines, especially if lines could be identified that express alternative HERV transcripts to 293 cells and can support PERV replication.

It is likely that the sequence divergence between PERV and HERV affected the ability of HERV transcripts to be cross-packaged into PERV virions. Increased rates of cross-packaging tend to be observed if the two participating viruses have a high degree of nucleotide similarity, which is in part due to the greater compatibility of the Gag proteins of the nascent retroviral particle with the packaging sequences of the genomic RNAs (Katz and Skalka, 1990). Although relatively little is known about HERV packaging signals, because the overall sequence similarity between PERV and HERV is low, it is likely that their packaging sequences will also be divergent and will therefore interact only very inefficiently, if at all. In support of this hypothesis, previous studies have demonstrated that the RNAs of several HERV groups are not packaged to a detectable level into MLV-based particles (Patience et al., 1998b). Thus, our results are consistent with previous studies indicating that the efficiency of packaging of HERV transcripts into γ-retrovirus particles is very low.

Interestingly, we found that MLV-derived transcripts were packaged at relatively low efficiency by PERV particles (approximately one copy in 10^4 particles), despite the significant sequence similarity that exists between these two viruses. Thus, while our results confirm earlier studies that demonstrate that replication-competent PERV can rescue MLV-based vectors (Patience et al., 1997; Takeuchi et al., 1998; Wilson et al., 2000), they suggest that this interaction may be less efficient than previously thought. In contrast, it should be noted that the correlation between genetic similarity and the efficiency of cross-packaging does not always apply. For example, although the packaging sequences of murine leukemia virus and the murine retroelement VL30 are divergent at the nucleotide level, they both remain compatible with MLV Gag proteins (Torrent et al., 1994). Consequently, when murine MLV-based retroviral packaging cells are used to produce retroviral vectors, approximately 15% of particles contain VL30 RNAs (Patience et al., 1998b). Thus, it appears difficult to predict the likelihood of cross-packaging based solely on nucleotide sequence data. Nevertheless, from a xenotransplantation safety perspective it remains encouraging that we did not detect any cross-packaging of any of the γ-retrovirus-related HERV groups (HERV-H, -W, -R, -E, and ERV-9) nor the β-retrovirus-related HERV group, HERV-K.

The expression of packagable PERV genomes within the 293 cells might have affected the rate of packaging of HERV transcripts into PERV particles. If HERV transcripts are to be packaged into PERV particles, they must be able to compete with high levels of PERV RNA expressed in an infected cell. It might therefore prove informative to study HERV genome cross-packaging into PERV particles in the absence of packagable PERV genomes. Although more artificial, the removal of competing PERV genomes might allow for inefficient HERV cross-packaging events to be detected. Such a study could be achieved if PERV-based human packaging cells were developed, i.e., human cells

engineered to express high levels of PERV Gag and Pol proteins. Similarly, overexpression of individual HERV transcripts in such packaging cells might also reveal extremely rare cross-packaging events.

In summary, we could not detect the cross-packaging of several groups of HERV sequences into PERV particles when using sensitive RT-PCRs. While these results are reassuring, it might prove interesting to extend this study with investigations of the copackaging of further HERV groups and the production of PERV-based pseudotype packaging cell lines. Ultimately, the risk that PERV–HERV recombination represents in vivo can only be assessed following clinical xenotransplantation. However, our in vitro results suggest that the risk is likely to be minimal.

Materials and methods

Cells

The human cell lines HEK293 and HepG2 were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Baltimore, MD) supplemented with 10% fetal bovine serum. 293/PERV-A 14/220 cells were produced following the coculture of miniature swine PBMC with uninfected 293 cells. The PERV-A isolate present in this culture contains recombinant PERV with the *gag*, *pol,* and *env* SU regions of PERV-A, with the TM region of PERV-C.

Sucrose density gradients

The culture supernatant from approximately 4×10^6 cells (at confluency) was collected over a 16-h period, 0.45-μm filtered, and fractionated by centrifugation through 12 ml linear sucrose density gradient (20 to 65%) at 100,000 *g* and 4°C for 16 h in an SW41 rotor (Sorval, Chicago, IL). The gradients were collected in 500 μl serial fractions prior to analysis for density determination, reverse transcriptase activity, and the presence of viral RNA transcripts by RT–PCR. The density of the gradient fractions was determined using a refractometer (Atago, Kirkland, WA).

Reverse transcriptase activity assays

The RT activity of gradient fractions was determined using an ELISA-based assay (HS-kit Mn2, Cavidi Tech AB, Sweden) in accordance with the manufacturer's recommendations. RT assessment was performed on 1:100 dilutions of the gradient fraction.

RNA expression

DNA-free RNA was purified from cells and sucrose gradient fractions using Trizol (Invitrogen Life Technologies) following the manufacturer's instructions. The purity of the RNA was confirmed by PCR analysis of RNA that had not been subjected to reverse transcription. Quality of the RNA was confirmed by amplification of either GAPDH or PERV *pol* sequences as appropriate. Random-primed cDNA was prepared using Superscript Kit (Invitrogen Life Technologies). Following cDNA synthesis, PCR amplification was performed on a 9600 thermal cycler (Perkin–Elmer, Atlanta, GA). PCRs were performed with 30 cycles of 94°C for 10 s, at 50°C 45 s, and 72°C for 30 s, with the exception of the HERV-K PCR that used an annealing temperature of 55°C. All oligonucleotide primers are listed 5′–3′. PCR products were visualized by ethidium bromide–agarose gel electrophoresis.

For Northern blot analysis of HERV mRNA transcripts, 15 μg of 293 cell total RNA was resolved on a 1% denaturing gel (0.6 M formaldehyde) in 1 × MOPS, 0.2 M formaldehyde running buffer. Neutral transfer of RNA was performed in 10× SSC using the Turboblotter (Schleicher & Schuell, Keene, NH) and Nytran Supercharge nylon transfer membranes (Schleicher & Schuell) according to the manufacturer's guidelines. Prehybridization and hybridization of membranes was performed at 42°C using the ULTRAhyb hybridization buffer (Ambion, Houston, TX) according to the manufacturer's guidelines. The membrane was sliced into a series of strips of equal size and RNA loading, and each strip was probed using PCR-generated ^{32}P-labeled probes derived by standard methodologies from the RT-PCR amplicons produced using the primers and conditions described above. The membranes were washed twice for 30 min at 65°C with 0.1× SSC/0.2% SDS and exposed to autoradiograph film for 72 h at −70°C.

RT-PCR sensitivity determination

ERV amplicons derived from 293 cell RNA were cloned into pTOPO-TA (Invitrogen Life Technologies). The orientation of the clones was determined and the constructs linearized adjacent to the most 3′ portion of the ERV insert with *Not*I or *Bam*HI to prevent runoff transcripts during RNA synthesis. The linearized plasmids were gel purified prior to the production of DNA-free RNA by SP6/T7 in vitro transcription in accordance with the manufacturer's instructions (Maxiscript kit, Ambion). The quality of the RNA was confirmed by gel electrophoresis and quantification against RNA of known concentration (Invitrogen Life Technologies) by ethidium bromide–ammonium acetate gel spotting. Known amounts of the transcribed RNA were titrated into the RT-PCR mixtures, enabling the sensitivity of the assays to be determined. The combination of these data, along with the dilution to which test samples could be taken and still provide a positive RT-PCR signal, allowed calculation of the concentration of RNA in the test samples.

Phylogenetic analysis and GenBank accession numbers

A neighbor-joining Phylogenetic analysis was performed using the following GenBank sequences: PERV (AF038599), HERV-E (M10976), HERV-R (M12140),

ERV-9 (X57147), HERV-H (K01891), HERV-W (AF135487), and HERV-K (M14123). The Phylip sequence analysis package was used to create the phylogenetic tree.

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